

Autophosphorylation of Type II CaM Kinase in Hippocampal Neurons: Localization of Phospho- and Dephosphokinase with Complementary Phosphorylation Site-Specific Antibodies

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Submitted October 15, 1992; Accepted December 9, 1992

We have visualized the distribution of autophosphorylated type II CaM kinase in neural tissue with the use of two complementary antibodies: a monoclonal antibody that binds to the α and β subunits of the kinase only when they are autophosphorylated at threonine-286 (287 in β) and affinity-purified rabbit antibodies that bind to both subunits only when they are not phosphorylated at these residues. We used these antibodies to double-label organotypic hippocampal cultures, detecting the mouse monoclonal antibody with rhodamine and the rabbit polyclonal antibodies with fluorescein. In double-exposed photographs, the ratios of intensities of the two fluorophores revealed the relative proportion of autophosphorylated and nonphosphorylated kinase in individual neurons throughout the cultures. We found that autophosphorylated and nonphosphorylated kinase are colocalized throughout most neurons rather than segregated within distinct cells or subcellular domains. However, the variations in intensity of the two fluorophores indicated that the proportion of autophosphorylated kinase is consistently higher in neuronal somas than in the neuropil. Incubation of the cultures in Ca^{2+} free medium dramatically reduced both the level of autophosphorylated kinase detected biochemically and the relative intensity of fluorescent staining with the phosphokinase specific monoclonal antibody. These results support the hypothesis that regulation of Ca^{2+} -independent CaM kinase activity in vivo occurs by a dynamic equilibrium between autophosphorylation and dephosphorylation and that this equilibrium is maintained, at varying steady-state levels, in all parts of neurons.

INTRODUCTION

Type II CaM kinase is the most abundant protein kinase in the hippocampus and forebrain, comprising $\sim 1\%$ of total forebrain protein (Erondy and Kennedy, 1985; Hunter, 1987). It is present in neuronal somas and dendrites (Ouimet *et al.*, 1984; Erondy and Kennedy, 1985) and is found in the cytosolic, membrane, and cytoskeletal fractions of brain homogenates (Stull *et al.*, 1986; Kennedy *et al.*, 1987). In the hippocampus, it plays a role in the initiation of long-term potentiation, a syn-

aptic phenomenon that may underlie early stages of memory formation (Kennedy, 1989; Malinow *et al.*, 1989; Squire and Zola-Morgan, 1991; Silva *et al.*, 1992a,b). The CaM kinase is the major component of the postsynaptic density fraction where it comprises 20–30% of total protein (Kennedy *et al.*, 1983, 1990; Miller and Kennedy, 1985). CaM kinase purified from the forebrain appears to be a heteromultimer ($M_r = 600\,000$ – $700\,000$) composed primarily of homologous α ($M_r = 54\,000$) and β ($M_r = 60\,000$) catalytic subunits (Bennett *et al.*, 1983; Goldenring *et al.*, 1983; Bennett and Kennedy, 1987; Lin *et al.*, 1987; Bulleit *et al.*, 1988). The activity of purified CaM kinase is dependent on Ca^{2+} and calmodulin, and it phosphorylates a broad range of brain substrate proteins when it is activated, including synapsin I, tryptophan hydroxylase, tyrosine

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hydroxylase, the microtubule-associated protein MAP2, and nitric oxide synthase (Stull *et al.*, 1986; Kennedy *et al.*, 1987; Colbran *et al.*, 1989; Bredt *et al.*, 1991). Phosphorylation of synapsin I by the CaM kinase facilitates exocytosis of synaptic vesicles (Llinas *et al.*, 1985; Nichols *et al.*, 1990). Phosphorylation of tyrosine hydroxylase has been correlated with a several-fold increase in its catalytic activity (Griffith and Schulman, 1988; Waymire *et al.*, 1988). Phosphorylation of MAP2 by the CaM kinase inhibits microtubule assembly in vitro (Yamamoto *et al.*, 1983). Thus, the CaM kinase may perform many regulatory functions associated with activity-dependent or hormone-induced increases in intracellular free Ca^{2+} .

The CaM kinase can become independent of Ca^{2+} /calmodulin after autophosphorylation of a threonine residue adjacent to the calmodulin-binding domain (threonine-286 in α and threonine-287 in β). The threonine residue is rapidly autophosphorylated after activation by Ca^{2+} /calmodulin (Miller *et al.*, 1988; Schworer *et al.*, 1988; Theil *et al.*, 1988). The presence of phosphate on this residue maintains the kinase in an active state, although at a reduced rate, after free Ca^{2+} is removed by chelation with EGTA (Miller *et al.*, 1988; Hanson *et al.*, 1989). The activation is highly cooperative; phosphorylation of only one to three subunits per dodecameric holoenzyme produces maximal Ca^{2+} -independent activity (Miller and Kennedy, 1986; Lickteig *et al.*, 1988). It has been postulated that this mechanism may allow the kinase to act as a switch in vivo by prolonging its activation beyond the duration of a transient increase in Ca^{2+} concentration (Miller and Kennedy, 1986; Lisman and Goldring, 1988). Phosphorylation of threonine-286/287 also enhances the affinity of the kinase for calmodulin, perhaps influencing the distribution of the calmodulin pool (Meyer *et al.*, 1992). We are interested in learning how this autophosphorylation is regulated in situ in hippocampal neurons.

Recent biochemical studies with neuronal cultures demonstrated that a significant basal level of autophosphorylated Ca^{2+} -independent CaM kinase activity is maintained in living neurons; however, the proportion of kinase that is autophosphorylated at basal Ca^{2+} concentration varies in different neuronal preparations. In dissociated cultures, the basal proportion of total kinase in the autophosphorylated state is 4–6% (Fukunaga *et al.*, 1989; Jefferson *et al.*, 1991), and in acute hippocampal slices, it is 7–9% (Molloy and Kennedy, 1991; Ocorr and Schulman, 1991). In neurons of organotypic hippocampal cultures, however, the basal proportion of total kinase in the autophosphorylated state is substantially higher, ~30%. This high basal autophosphorylation is not caused by elevated cytosolic Ca^{2+} because the basal Ca^{2+} concentration in these neurons is 30–45 nM (Molloy and Kennedy, 1991). Furthermore, suppression of electrical activity in the cultures by pro-

longed application of tetrodotoxin or glutamate receptor antagonists does not decrease the basal level of autophosphorylation. The proportion of autophosphorylated kinase in homogenates of dissected hippocampi is intermediate between the level in homogenates of acute slices and that in homogenates of organotypic cultures; it varies from 13–20% depending on developmental age (Molloy and Kennedy, 1991). It is not yet clear which of these neuronal preparations best reflects the proportion of basal autophosphorylated CaM kinase in intact hippocampi.

The basal Ca^{2+} -independent CaM kinase activity appears to be maintained by a dynamic equilibrium between autophosphorylation and dephosphorylation. Thus, in organotypic hippocampal cultures, Ca^{2+} -independent CaM kinase activity can be dramatically increased by inhibiting phosphatase activity and reduced by lowering the external Ca^{2+} concentration (Molloy and Kennedy, 1991). Therefore, it appears that an important function of the autophosphorylation mechanism in hippocampal neurons may be to produce a relatively high level of CaM kinase activity, even at basal Ca^{2+} concentrations, permitting both upward and downward local regulation by physiological agents. The CaM kinase may function more like an analogue “dimmer” switch than a digital switch.

One serious difficulty in interpreting these results has been that available biochemical methods do not allow one to determine whether the level of CaM kinase autophosphorylation is uniform throughout the neurons in the cultures or higher in particular subpopulations of neurons or subcellular compartments of neurons. The most extreme version of the “switch” hypothesis would predict that each individual neuron would contain either CaM kinase that is fully autophosphorylated or CaM kinase that is completely dephosphorylated (Lisman, 1985). Thus, autophosphorylated kinase would be located primarily in a subset of neurons; e.g., 30% of neurons in the case of organotypic cultures. Similarly, it is not possible to determine experimentally whether biochemically measured changes in autophosphorylation produced by pharmacological agents or electrical stimulation reflect changes in the total pool of kinase or changes localized to discrete neurons, areas of neurons, or synaptic regions. In this study, we used antibodies specific for the phospho- or nonphosphokinase to visualize the distribution of autophosphorylated CaM kinase in fixed sections of organotypic cultures.

MATERIALS AND METHODS

Preparation of Nonphosphorylated and Thiophosphorylated Peptide Immunogens

A 14-residue peptide with the sequence MHRQETVDCLKKFN (residues 281–294 of the α subunit) was synthesized by the Biopolymer Facility at Caltech. Nonphosphopeptide immunogen was prepared

by first purifying the peptide by reverse-phase high-performance liquid chromatography to >95% purity and then coupling the peptide through the cysteine residue to keyhole limpet hemocyanin (KLH; Sigma Chemical, St. Louis, MO; 10 mg) with succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC; Pierce Chemical, Rockford, IL). Thiophosphorylated peptide immunogen was prepared by enzymatically thiophosphorylating 30 mg of peptide in a reaction mixture containing 50 mM tris(hydroxymethyl)aminomethane (Tris) (pH 8.0), 10 mM MgCl₂, 0.4 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.7 mM CaCl₂, 20 mM dithiothreitol, 10 mg/ml peptide (5.7 mM), 7.0 mM ATP γ S, 0.1 mg/ml calmodulin, and 0.1 mg/ml CaM kinase purified by the method of Miller and Kennedy (1985) in a final volume of 3.0 ml. The reaction was carried out at 30°C for 5 h. The reaction mix was injected onto a semipreparative reverse-phase C18 column (Vydac, Hesperia, CA), and thiophosphorylated peptide was purified as previously described (Patton *et al.*, 1991). The molar yield from starting material was 33% (10.5 mg). Thiophosphorylated peptide (10 mg) was coupled through the cysteine residue to 10 mg KLH preactivated with SMCC. KLH-conjugated peptide (5 mg protein/ml) was aliquoted without further purification and stored at -80°C.

Selection of a Monoclonal Antibody Specific for Phosphorylated CaM Kinase

BALB/c ByJ mice (aged 6 wk; Jackson Labs, Bar Harbor, ME) were primed and twice boosted at 3-wk intervals by intraperitoneal injection of a mixture of KLH-conjugated thiophosphorylated peptide (100 μ g KLH) and free thiophosphorylated peptide (~90 μ g), emulsified in adjuvant (Ribi ImmunoChem Research, Hamilton, MT) as described previously (Patton *et al.*, 1991). Test bleeds were obtained 7 d after each injection. A final series of boosts was performed without adjuvant on the 3 d preceding the fusion (Stähli *et al.*, 1980). Splenocytes from one of the mice were fused with HL-1 murine myeloma cells (Ventrex, Portland, ME). Sera from test bleeds and media collected from hybridoma cultures were screened for antibodies against autophosphorylated CaM kinase by enzyme-linked immunosorbent assay (ELISA) with nonphosphorylated and autophosphorylated CaM kinase as antigen as described previously (Patton *et al.*, 1991). CaM kinase used as antigen in the ELISA was autophosphorylated with ATP in place of ATP γ S. Clones testing positive against phosphokinase and negative against nonphosphokinase were subcloned at clonal density, and Ascites tumors were produced in pristane-primed BALB/c mice (Simonsen, Gilroy, CA). Antibody was partially purified from Ascites fluid by ammonium sulfate precipitation (50% saturation), resuspended in phosphate-buffered saline (PBS) to 30 mg/ml, and stored at -80°C. Three monoclonal antibodies produced by this fusion are described in this article: 22B1, 26G6, and 27G10.

Preparation of a Polyclonal Antiserum Specific for Nonphosphorylated CaM Kinase

Female New Zealand white rabbits (ABC Rabbitry, Chino, CA) were immunized by priming and boosting with a mixture of KLH-conjugated peptide MHRQETVDCLKKFN (0.6 mg KLH) and free peptide (~0.5 mg), emulsified in adjuvant (RIBI ImmunoChem Research), as described (Patton *et al.*, 1991). Rabbits were injected at multiple subcutaneous and intramuscular sites and boosted four to six times at intervals of 6–10 wk. Serum taken 1 wk after each boost was tested for antibodies that selectively bound to nonphosphorylated CaM kinase on immunoblots. Antibodies specific for the nonphosphorylated kinase were purified by ammonium sulfate precipitation and affinity chromatography. One antiserum produced by this procedure is described in this article and is referred to as "Sylvia" antiserum.

Affinity Purification of Rabbit Antisera

Affinity resin was produced by coupling the nine residue synthetic peptide MHRQETVDC (residues 281–289 of the α subunit) to

SulfoLink agarose-CL resin (Pierce Chemical, Rockford, IL) according to the manufacturer's directions. Peptide (1 mg/ml resin) was coupled to the resin with nearly 100% efficiency. The affinity resin was preabsorbed with nonimmune rabbit serum (GIBCO, Grand Island, NY) before use. Immune antiserum was precipitated with ammonium sulfate (50% saturation), and the pellet was resuspended in 20 mM sodium phosphate (pH 7.2), 9% (wt/vol) NaCl (PBS) to one-half the original serum volume. This antibody solution (4 ml) was diluted fivefold with binding buffer (50 mM Tris [pH 7.5], 5 mM EDTA) and stirred gently with the affinity resin (1 ml) for 3 h at 4°C. The mixture was poured into a column, and unbound antibodies were washed from the resin with 10 column volumes of binding buffer. Bound antibodies were eluted with a commercial buffer (Gentle Elution Buffer; Pierce Chemical). Fractions containing protein detected by absorbance at 280 nm were dialyzed against 20 mM sodium phosphate (pH 7.4) at 4°C and assayed for nonphosphokinase-specific antibody by immunoblot and ELISA. The final antibody concentration was estimated by absorbance at 280 nm.

Immunoblots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Samples containing 0.2 μ g CaM kinase holoenzyme or 20 μ g brain homogenate protein were electrophoresed in minigels (5 \times 9 \times 0.08 cm) containing 10% polyacrylamide and 0.34% bis-acrylamide. Gels were washed for 20 min in H₂O, and then protein was electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) in 25 mM sodium borate for 2.5 h at 350 mA. Membranes were blocked with 5% (wt/vol) nonfat dry milk in 50 mM Tris (pH 7.4), 0.5 M NaCl, and 0.05% (vol/vol) Tween 20 (Bio-Rad Laboratories, Richmond, CA) (TBS-Tween) and then incubated with primary antibody diluted in TBS-Tween supplemented with 2% (vol/vol) normal goat serum (GIBCO) for 12 h at 4°C. Bound antibodies were detected by incubation for 2 h at room temperature with goat anti-mouse or goat anti-rabbit IgG + IgM secondary antibodies conjugated with alkaline phosphatase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) diluted 2000- or 4000-fold. Bound secondary antibodies were visualized by reaction with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and *p*-nitro blue tetrazolium chloride alkaline phosphatase substrates (Bio-Rad) dissolved in 0.1 M NaHCO₃ (pH 9.5), 1 mM MgCl₂.

Transfection of COS 7 Cells

A full-length cDNA encoding the rat brain CaM kinase α subunit was a generous gift of Dr. R.F. Bulleit (Bulleit *et al.*, 1988). α Subunit cDNA sequences in which the threonine-286 codon was mutated to Ala or Asp were a generous gift from Dr. M. Ito (Caltech). The mutants were generated by a polymerase chain reaction initiated with a degenerate oligonucleotide primer designed to introduce mutations at nucleotide positions 856 and 857 of the α subunit sequence. The mutant reaction products were substituted into the full-length α subunit cDNA, and the final products were sequenced to verify the mutant constructs. Mutant α subunit cDNAs were cloned into pcDL-SR α 2, a COS cell expression vector constructed by Dr. R.F. Bulleit, as previously described (Cho *et al.*, 1991). Before transfection, 4 \times 10⁶ COS 7 cells were transferred to a 60-mm plate and grown for 24 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Cells were transfected with pcDL-SR α 2 vector DNA containing wild-type and mutant α subunit sequences by the DEAE-dextran method (Luthman and Magnusson, 1983; Lopata *et al.*, 1984) and grown for 3 d, at which time they were harvested, homogenized by sonication in ice-cold buffer (20 mM Tris, 1 mM imidazole [pH 7.5], 1 mM dithiothreitol, 1 mM MgCl₂, 20 mM sodium pyrophosphate, 25 mg/ml soybean trypsin inhibitor [Sigma], 1 μ M leupeptin [Boehringer Mannheim], and 0.25 mM phenylmethylsulfonyl fluoride [Sigma]), and centrifuged for 15 min at 13 000 \times g. Supernatants containing expressed α subunits were stored at -80°C.

Preparation of Organotypic Cultures

Organotypic cultures of rat hippocampal slices were prepared by a modification of the method of Gähwiler (1984), as previously described (Molloy and Kennedy, 1991). Briefly, hippocampi dissected from 4- to 6-d-old Sprague-Dawley rats (Simonsen) were sliced into 400- μ M parasagittal sections with a 20- μ m wire grid and incubated at 4°C in Gey's balanced salt solution for 30 min. Slices were then immobilized on glass coverslips with Vitrogen (Celtrix, Palo Alto, CA) and cultured in tubes containing 1 ml of medium (Gähwiler, 1984) placed in a roller drum. Cultures were treated with antimetabolic agents to inhibit glial cell proliferation, as described previously (Molloy and Kennedy, 1991). The cultures were fixed and embedded after ~4 wk in culture.

Pretreatment, Fixation, and Preparation of Semithin Sections of Organotypic Cultures

Organotypic hippocampal cultures were either transferred directly to cold fixative solution or were first preincubated in Ca^{2+} -free saline solution (25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [pH 7.4], 26 mM NaHCO_3 , 1.25 mM NaH_2PO_4 , 125 mM NaCl, 2 mM KCl, 2 mM MgSO_4 , 10 mM glucose, and 0.2 mM EGTA) for 30 min at 37°C. Cultures were fixed in place on the coverslip for 1 h at 0°C with 4% (wt/vol) paraformaldehyde and 0.2% (wt/vol) glutaraldehyde in 0.1 M cacodylate (pH 7.2) containing 15 mM MgCl_2 and 100 μ M 2-amino-5-phosphonopentanoic acid. Fixed cultures were washed with 0.1 M cacodylate (pH 7.2) and postfixed in 0.04% osmium tetroxide for 1 h at 0°C. Cultures were then washed in 0.1 M sodium phosphate (pH 7.4) and dehydrated by successive incubations in 70, 95, and 100% ethanol. Dehydrated cultures were impregnated with EM bed-812 resin (EM Sciences, Fort Washington, PA) at 0°C over 3 h with two changes of resin without activator. Cultures were scraped from the cover slips at the first change in resin. Cultures were incubated overnight at room temperature in resin containing activator (1.5% [vol/vol] 2,4,6-tri(dimethylaminomethyl)phenol [DMP-30]). After two final changes of resin containing DMP-30, the cultures were incubated at 45°C for 90 min. Each culture was then mounted in resin onto a chunk of prehardened resin and flattened against a glass slide with the aid of a spring-loaded press. The resin was hardened for 20 h at

65°C. After trimming to expose the face of the embedded hippocampal slice, each block was positively identified by nicking the edge of the block face. Cultures were then sectioned at 2 μ M on an ultra microtome. Sections from control and experimental cultures were identified by their nicked edges and were mounted in pairs on the same slide. In this way, sections from different hippocampal cultures, pretreated in different ways, could be stained together.

Immunofluorescence Labeling and Microscopy

Sections were etched for 4 min with 13.3% (wt/vol) KOH in 33% (vol/vol) propylene oxide, 67% (vol/vol) absolute methanol. They were then blocked with 0.1 M glycine (pH 7.2) for 30 min, rinsed briefly with H_2O , and reactive aldehydes were reduced with 1% (wt/vol) NaBH_4 for 10 min. After brief rinses with H_2O and 20 mM sodium phosphate (pH 7.2), 0.45 M NaCl, 0.1% (vol/vol) Triton-X 100 (PBS-Triton buffer), sections were blocked for 45 min with 5% normal goat serum in PBS-Triton buffer (blocking buffer) and then incubated with primary antibody diluted in blocking buffer for 12–18 h at 4°C. Antibodies 6G9, 22B1, and affinity-purified Sylvania were diluted to 0.08, 0.014, and 0.06 mg/ml, respectively. After washing with blocking buffer, sections were incubated for 1 h at room temperature with fluorescein- and/or rhodamine-conjugated secondary antibodies directed against rabbit or mouse IgG, respectively (Cappel, Organon Teknica, Durham, NC) diluted 100-fold in blocking buffer. After several 10-min washes in blocking buffer and one wash in 5 mM phosphate buffer, sections were mounted in 50% glycerol containing 10 mg/ml *p*-phenylenediamine. They were viewed by epifluorescence through barrier filters (Olympus, Lake Success, NY) for either rhodamine (BP545) or fluorescein (BP490 and EY455), and images were recorded on color reversal film (P800/1600; Kodak, Rochester, NY). Within an experiment, all images were taken with identical exposure settings to allow comparison of ratios of fluorescence intensity among different regions and sections. Double immunofluorescence images were obtained by double exposure with rhodamine and fluorescein filter sets.

Other Materials and Methods

Calmodulin was purified by the method of Watterson *et al.* (1976). Protein was determined by the method of Peterson (1977) with bovine

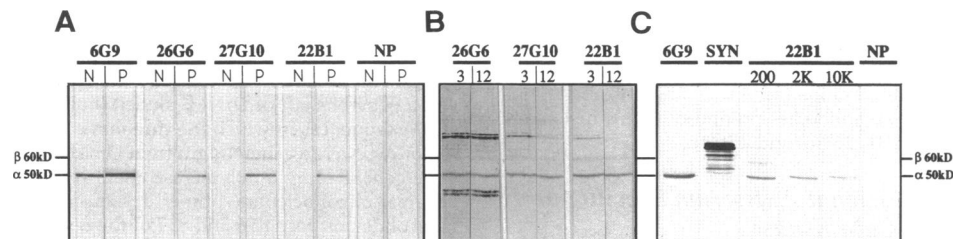


Figure 1. Specificity of monoclonal antibodies for autophosphorylated CaM kinase on immunoblots. Purified kinase was autophosphorylated *in vitro* as described (Miller *et al.*, 1988) for 2 min at 30°C either in the presence or absence of 200 μ M [γ - ^{32}P]ATP. Reactions contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl_2 , 0.9 mM CaCl_2 , 0.6 mM EGTA, 10 mM dithiothreitol, 0.2 mg/ml calmodulin, and 0.2 mg/ml kinase holoenzyme. Rat brain homogenate protein was prepared as described (Bennett *et al.*, 1983) and phosphorylated in the presence of Ca^{2+} by endogenous kinases *in vitro* for 2 min at 30°C as described above, except that reactions contained 1 mg/ml homogenate protein, 50 μ g/ml calmodulin, and 100 μ M [γ - ^{32}P]ATP. Phosphorylated proteins (0.2 μ g CaM kinase; 20 μ g homogenate protein) were separated by SDS-PAGE and transferred to nitrocellulose as described under MATERIALS AND METHODS. After autoradiography, lanes were cut from the blots and incubated with the indicated monoclonal antibody. Bound antibody was detected with alkaline phosphatase-conjugated secondary antibody as described under MATERIALS AND METHODS. The positions of kinase α and β subunits are indicated at the margins. (A) Lanes containing purified nonphosphorylated (N) or phosphorylated (P) CaM kinase were incubated for 12 h with antibodies 6G9 (to label α subunit), 26G6, 27G10, or 22B1 diluted 2000-fold from ammonium sulfate purified ascites (30 mg/ml). Adjacent control lanes were incubated without primary antibody (NP). (B) Lanes containing phosphorylated homogenate protein were incubated for 12 h with antibodies 26G6, 27G10, or 22B1 diluted 500-fold. The blots were washed for either 3 or 12 h with several changes of buffer before detecting bound antibody. (C) Lanes containing phosphorylated homogenate protein were incubated for 20 h with antibody 22B1 diluted 200, 2000 (2K), or 10 000-fold (10K). Adjacent control lanes were incubated with 6G9 (1/2000), polyclonal antisera directed against synapsin I (SYN; 1/10 000), or no primary antibody (NP). Blots were washed for 1 h before detection with secondary antibody.

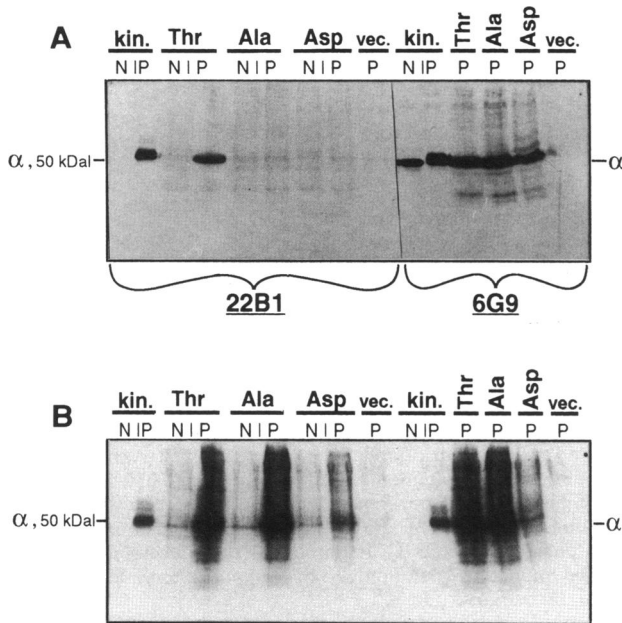


Figure 2. Specificity of monoclonal antibody 22B1 for autophosphorylated threonine-286. COS 7 cells were transfected with expression vector DNA containing either the wild-type sequence of rat CaM kinase α subunit encoding threonine at residue 286 (Thr) or mutated sequences encoding alanine (Ala) or Aspartate (Asp) at residue 286. Control transfections with vector DNA lacking an insert were carried out in parallel. COS cell homogenates containing wild-type kinase, mutants, or vector alone were phosphorylated in the presence (P) or absence (N) of Ca^{2+} /calmodulin (0.1 mg/ml) as described in Figure 1. Reactions contained 0.3 mg/ml homogenate protein, 200 μM [γ - ^{32}P]ATP (1200–1400 cpm/pmol) and were carried out for 3 min at 30°C. Purified CaM kinase holoenzyme (kin) was autophosphorylated (P) for 1 min in the presence of Ca^{2+} , calmodulin, and [γ - ^{32}P]ATP. Nonphosphorylated kinase holoenzyme (N) was prepared identically except that ATP was omitted from the reaction. Proteins (0.1 μg kinase holoenzyme; 16 μg homogenate) were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibody 22B1 or 6G9, each at 1000-fold dilution. (A) Immunoblot of lanes 1–9 with 22B1 and lanes 10–15 with 6G9. (B) Autoradiogram of A. The position of the kinase α subunit is indicated in the margins.

serum albumin as standard. Annette antiserum, raised against fore-brain holoenzyme, recognizes both the α and β subunits of the kinase. The monoclonal antibody 6G9 was partially purified from Ascites fluid by ammonium sulfate precipitation; it specifically recognizes the CaM kinase α subunit (Erond and Kennedy, 1985). Tryptic phosphopeptide maps were prepared as previously described (Miller *et al.*, 1988; Patton *et al.*, 1990; Molloy and Kennedy, 1991).

RESULTS

Isolation of a Monoclonal Antibody Specific for CaM Kinase Autophosphorylated at Threonine-286

Mice were immunized with a conjugated thiophosphorylated 14 residue peptide with the sequence MHRQET(PSO₃)VDCLKKFN as described under MATERIALS AND METHODS. This sequence corresponds to the sequence surrounding threonine-286 in the α

subunit; phosphorylation of threonine-286 controls Ca^{2+} -independent kinase activity. To minimize dephosphorylation of the peptide after its injection into mice and thereby enhance the specificity of the immune response for phosphorylated peptide, we chose to thiophosphorylate the peptide because thiophosphorylated serine and threonine residues are resistant to dephosphorylation by most cellular protein phosphatases (Gratecos and Fischer, 1974; Cassel and Glaser, 1982). Of 1248 hybridoma cultures (average of three clones per well), 24 clones (2%) secreted antibody that reacted at least 10 times more strongly with autophosphorylated CaM kinase than with nonphosphorylated kinase measured by ELISA. Antibodies from approximately half of these clones reacted only with the autophosphorylated form of CaM kinase on immunoblots and did not react with nonphosphorylated kinase (e.g., 26G6, 27G10, and 22B1; Figure 1A). Most of them bound more tightly to the α subunit ($M_r = 50\,000$) than to the β subunit ($M_r = 60\,000$).

To determine whether these monoclonal antibodies are specific for the CaM kinase, blots of total brain homogenates were prepared (Figure 1). Several of the antibodies recognized proteins in brain homogenates other than the CaM kinase (Figure 1B). The most common cross-reactive protein was phospho-synapsin I, which contains two phosphorylation sites for the CaM kinase that are similar in sequence to the peptide immunogen. Synapsin I is visible as a doublet at apparent $M_r = 80\,000$ in Figure 1B and in the second lane of Figure 1C. However, the most avid and specific monoclonal antibody, 22B1, did not cross-react with synapsin I or other brain proteins on immunoblots when the blots were washed for 12 rather than 3 h (Figure 1B) or when antibody was applied at appropriately high dilution (Figure 1C).

Threonine-286 is the autophosphorylation site that regulates Ca^{2+} -independent activity (Miller *et al.*, 1988; Hanson *et al.*, 1989). However, the kinase is also autophosphorylated at several other sites (Miller *et al.*, 1988; Patton *et al.*, 1990). To verify that 22B1 recognizes only the autophosphorylated site at threonine-286, we tested its ability to bind, before and after autophosphorylation, to α subunits containing point mutations at position 286. Wild-type α subunits and α subunits containing alanine or aspartate in place of threonine-286 were expressed in COS 7 cells. The α subunits in homogenates of the transfected cells were autophosphorylated and blotted onto nitrocellulose as described under MATERIALS AND METHODS. Mutant and wild-type α subunits were both expressed as demonstrated by immunoblots with antibody 6G9 that recognizes both autophosphorylated and nonphosphorylated α subunit (Figure 2B) (Erond and Kennedy, 1985). Antibody 22B1 bound to autophosphorylated wild-type α subunit but did not bind to the autophosphorylated alanine-286 mutant (Figure 2A), although that mutant

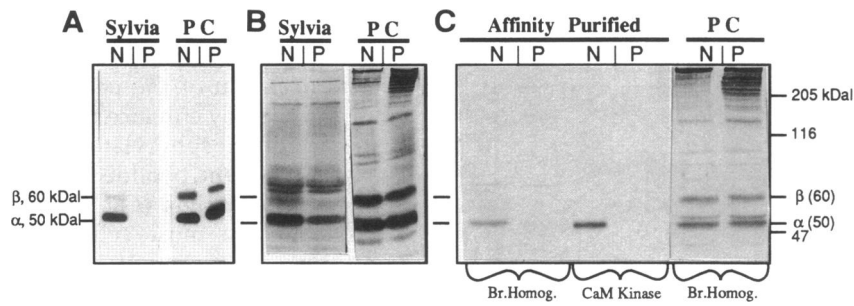


Figure 3. Specificity of affinity purified Sylvia antibodies for nonphosphorylated CaM kinase. Purified CaM kinase and brain homogenates were phosphorylated as described in Figure 1. Kinase was incubated in the presence (P) or absence (N) of ATP. Homogenates were incubated in the presence of ATP and in the presence (P) or absence (N) of Ca^{2+} /calmodulin. Proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. Adjacent strips were incubated with either Sylvia polyclonal antibodies or control antibody (PC), which consisted of a mixture of monoclonal antibody 6G9 (diluted 2000-fold) and polyclonal antibodies from Annette (diluted 500-fold), a rabbit immunized with purified CaM kinase holoenzyme. The control mixture recognizes both nonphosphorylated and autophosphorylated α and β subunits. (A) Immunoblots of purified kinase with Sylvia antibodies partially purified by precipitation with 50% saturated ammonium sulfate and diluted 250-fold. Binding of the antibodies is nearly completely blocked by autophosphorylation. (B) Immunoblots of endogenously phosphorylated brain homogenate protein with Sylvia antibodies as in A. Binding of the antibodies to the kinase subunits is reduced by Ca^{2+} /calmodulin-stimulated phosphorylation. Residual binding in the autophosphorylated lane may be due to incomplete phosphorylation. (C) Immunoblots of brain homogenate and purified CaM kinase with affinity purified Sylvia antibodies (1.9 $\mu\text{g}/\text{ml}$). Purified antibodies bound only to the nonphosphorylated α subunit. Positions of the kinase subunits and molecular weight markers are indicated in the margins.

incorporated phosphate at sites other than threonine-286 (Figure 2B, lanes 4 and 7). It also did not bind to the aspartate-286 mutant (Figure 2A, lane 8). Thus, antibody 22B1 specifically recognizes CaM kinase that is autophosphorylated on threonine-286. Further support for this conclusion comes from experiments in which antibody 22B1 bound nearly maximally to the purified kinase subunits after only 5 s of autophosphorylation, at which time threonine-286 is the only autophosphorylated residue (Miller *et al.*, 1988; Patton *et al.*, 1990).

Preparation of Rabbit Antisera Specific for CaM Kinase that is not Autophosphorylated at Threonine-286

Rabbits were immunized with the conjugated 14-residue peptide described in the previous section, but the peptide was not thiophosphorylated. Antisera from one rabbit, Sylvia, was highly specific for the nonphosphorylated form of the CaM kinase on immunoblots (Figure 3A), as previously described (Patton *et al.*, 1991). However, it cross-reacted with a number of other proteins present in rat brain homogenates (Figure 3B). Therefore, antibodies specific for the CaM kinase were purified by affinity chromatography on Agarose-CL resin conjugated with the 9-residue peptide, MHRQETVDC, as described under MATERIALS AND METHODS. The purified antibodies are specific for nonphosphorylated kinase and bind preferentially to the α subunit rather than the β subunit (Figure 3C). They cross-react slightly with a single protein of apparent $M_r = 65\,000$ in rat brain homogenates (Figure 3C). Binding to this protein is not affected by Ca^{2+} /

calmodulin-dependent phosphorylation (Figure 3C). Binding to both the kinase and to the 65-kDa protein is inhibited by preabsorption with the 9- or 14-residue synthetic peptides described above.

To establish that affinity-purified Sylvia antibodies recognize the CaM kinase only at the sequence surrounding threonine-286, we tested their ability to bind to the mutated α subunits described above. The antibodies bound to the wild-type α subunit and their binding was reduced by autophosphorylation (Figure 4A, lanes 1 to 4). The apparent residual binding to the autophosphorylated wild-type subunit may be due to incomplete autophosphorylation. The antibodies also bound to the alanine-286 mutant, but its autophosphorylation did not significantly reduce binding (Figure 4A, lanes 6 and 7). In contrast, the replacement of threonine-286 by aspartate completely blocked binding of the Sylvia antibodies to the α subunit (Figure 4A, lanes 8 and 9). This result indicates that aspartate, which has a large negative charge at neutral pH, can mimic a phosphate group at position 286 and block binding of Sylvia antibodies. Taken together, these results indicate that the affinity-purified Sylvia antibodies are specific for the nonphosphorylated form of the CaM kinase, and most of their binding to this form is at the threonine-286 autophosphorylation site. This conclusion is supported by the additional observation that 5 s of autophosphorylation of purified CaM kinase almost completely blocks binding of the Sylvia antibodies.

Distribution of Autophosphorylated CaM Kinase II in Organotypic Cultures of Hippocampal Slices

We first labeled sections of organotypic cultures with either the phosphokinase-specific 22B1 monoclonal an-

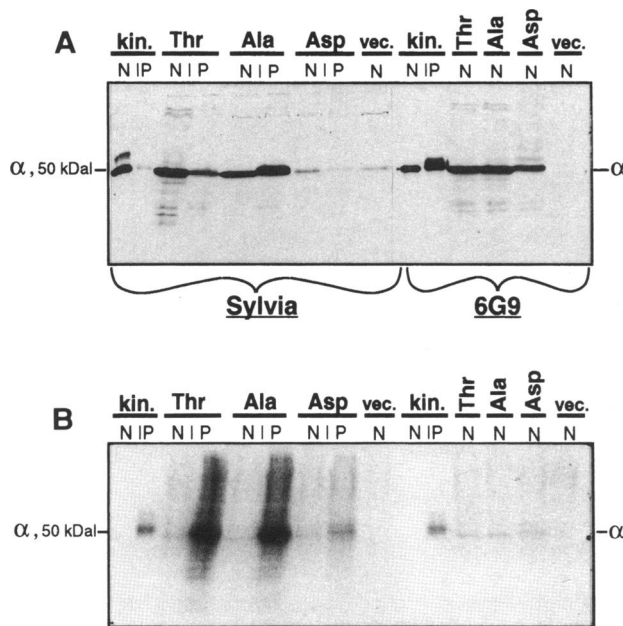


Figure 4. Specificity of affinity purified Sylvia polyclonal antibody for the Thr286 autophosphorylation site. COS 7 cells were transfected with expression vector plasmid DNA containing either the wild-type sequence of the α subunit (Thr) or mutated sequences encoding alanine (Ala) or Aspartate (Asp) at residue 286. Control transfections were carried out in parallel with vector DNA lacking the subunit insert. Cell homogenates containing wild-type kinase, mutants, or vector alone were phosphorylated for 3 min in the presence (P) or absence (N) of Ca^{2+} /calmodulin as described in Figure 2 and in MATERIALS AND METHODS. Nonphosphorylated (N) and autophosphorylated (P) purified CaM kinase holoenzyme (kin) was prepared as in Figure 2. Proteins (0.1 μg kinase holoenzyme; 16 μg homogenate) were fractionated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with Sylvia antibodies or monoclonal antibody 6G9. (A) Immunoblot of lanes 1–9 with affinity-purified Sylvia antibodies (0.6 $\mu\text{g}/\text{ml}$) and lanes 10–15 with 6G9 (diluted 1000-fold). (B) Autoradiogram of A. The position of the α subunit is indicated in the margins.

tibody or the nonphosphokinase-specific Sylvia antibodies. The pattern of labeling with these antibodies was compared with that of monoclonal antibody 6G9, which binds to the CaM kinase α subunit in both the autophosphorylated and nonphosphorylated state (Figure 1). All three antibodies labeled most of the neurons in the cultures (Figure 5). The distribution of label was similar for all three: cell somas and neuropil areas were densely labeled and nuclei were lightly labeled. Thus, most neurons and subcellular domains contain both phospho- and nonphospho-CaM kinase.

We reasoned that double labeling with phospho- and nonphosphokinase specific antibodies, each detected with a different fluorophore, would be the most sensitive way to reveal small differences in distribution of phosphorylated and nonphosphorylated kinase. With this method, the intensity of one fluorophore would always decrease as the intensity of the other increases; there-

fore, the ratio of the two fluorescence intensities would be a sensitive function of the ratio of phospho- and nonphosphokinase. Sections were labeled with 22B1 mouse antibodies detected with rhodamine and Sylvia rabbit antibodies detected with fluorescein. Thus, we expected that regions of the cultures with relatively more phosphokinase would appear relatively orange in double exposures, whereas regions of the cultures with a lower proportion of autophosphorylated kinase would appear relatively green. Double exposures were made by choosing an appropriate exposure time for each fluorophore with the appropriate filters in place and exposing a single frame once with each filter set. The relative intensities of the fluorophores in double-exposures provide information about relative levels of autophosphorylation of the kinase among different regions in each section and among sections labeled at the same time. However, because the exposure times were optimized for the color film, the intensities do not provide information about absolute levels of autophosphorylation.

As we expected, the doubly exposed pictures revealed more variability in the distribution of autophosphorylated kinase than was apparent after labeling with either antibody alone (Figure 6). The variability was most apparent among a group of pyramidal cell somas near the CA3/CA1 border (arrows in Figure 6). A number of cells in this region were not labeled by the monoclonal antibody against phosphorylated kinase but were strongly labeled by the antibody against nonphosphorylated kinase. These cells, which number ≤ 2 –3% of the cells in the cultures, appear green and stand out in striking contrast to neighboring cells and to the adjacent molecular layer. In most sections, neuronal somas labeled more intensely with the anti-phosphokinase antibody than did the neuropil areas, and therefore appeared more orange in the double exposures (compare upper and lower arrowheads in Figures 6C and 8A). The results indicate that, in general, the basal autophosphorylated kinase that we previously detected by biochemical methods in organotypic cultures (Molloy and Kennedy, 1991) is distributed throughout most of the neurons. However, the autophosphorylated form is somewhat more concentrated in the soma than in dendrites and axons and is nearly absent from a small subpopulation of neurons.

Labeling of Organotypic Cultures with Anti-Phosphokinase Antibodies is Reduced after Removal of External Ca^{2+}

We previously found that incubation of organotypic hippocampal cultures for 30 min in a Ca^{2+} -free solution containing 200 μM EGTA substantially reduces the basal level of Ca^{2+} -independent CaM kinase activity (Molloy and Kennedy, 1991). To confirm that this reduction is

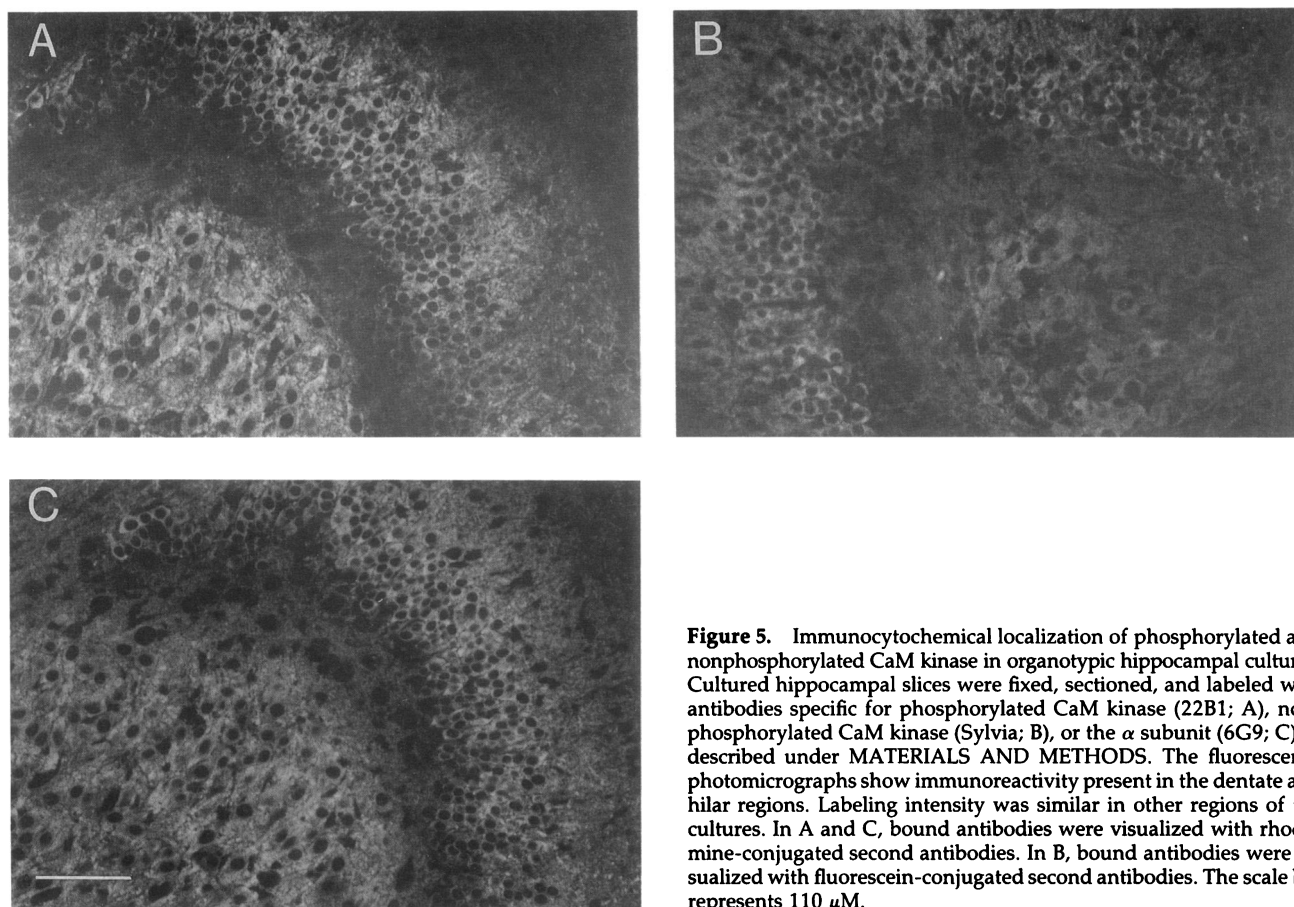


Figure 5. Immunocytochemical localization of phosphorylated and nonphosphorylated CaM kinase in organotypic hippocampal cultures. Cultured hippocampal slices were fixed, sectioned, and labeled with antibodies specific for phosphorylated CaM kinase (22B1; A), nonphosphorylated CaM kinase (Sylvia; B), or the α subunit (6G9; C) as described under MATERIALS AND METHODS. The fluorescence photomicrographs show immunoreactivity present in the dentate and hilar regions. Labeling intensity was similar in other regions of the cultures. In A and C, bound antibodies were visualized with rhodamine-conjugated second antibodies. In B, bound antibodies were visualized with fluorescein-conjugated second antibodies. The scale bar represents 110 μ M.

caused by decreased autophosphorylation of threonine-286, we labeled control cultures and cultures incubated in Ca^{2+} -free medium with $^{32}\text{PO}_4$ and then prepared phosphopeptide maps of the α subunit after immunoprecipitating it from culture homogenates. Maps prepared from control cultures (Figure 7A) contained several previously identified phosphopeptide peaks (Miller *et al.*, 1988; Patton *et al.*, 1990), including those containing threonine-286, whereas maps from the EGTA-treated cultures revealed a dramatic and specific reduction of ^{32}P -phosphate in the peptides that contain threonine-286 (Figure 7B).

To test whether this reduction in autophosphorylation of the kinase at threonine-286 occurs throughout all the neurons, we prepared sections of cultures that had been incubated in Ca^{2+} -free EGTA medium for 30 min and labeled them with the two antibodies (Figure 8). Control cultures that were incubated in medium containing 1.8 mM Ca^{2+} were processed in parallel and labeled with the same antibody solutions. Sections from the control cultures were used to determine exposure times for each fluorophore; then the same exposure times were used to photograph the

sections from EGTA-treated cultures. Double-exposures of the EGTA-treated cultures (Figure 8B) are much greener than the comparable control cultures (Figure 8A), indicating that staining with 22B1 is specifically reduced. In the experiment shown in Figure 8, nearly complete dephosphorylation of the CaM kinase was observed in both of two EGTA-treated cultures: staining with 22B1, the anti-phosphokinase antibody, was hard to detect whereas staining with Sylvia, the anti-nonphosphokinase antisera, was intense. In contrast, 22B1 and Sylvia both stained the two control cultures intensely. The level of phosphokinase immunoreactivity was also significantly reduced in EGTA-treated cultures in two additional experiments, but staining with 22B1 was not eliminated completely. Thus, a change in the relative intensity of staining with the complementary antibodies 22B1 and Sylvia correlates with a reduction in phosphorylation measured biochemically. Furthermore, the immunocytochemical staining reveals that the reduction of autophosphorylated kinase produced by incubation in EGTA occurs in all subcellular domains of neurons throughout the culture.

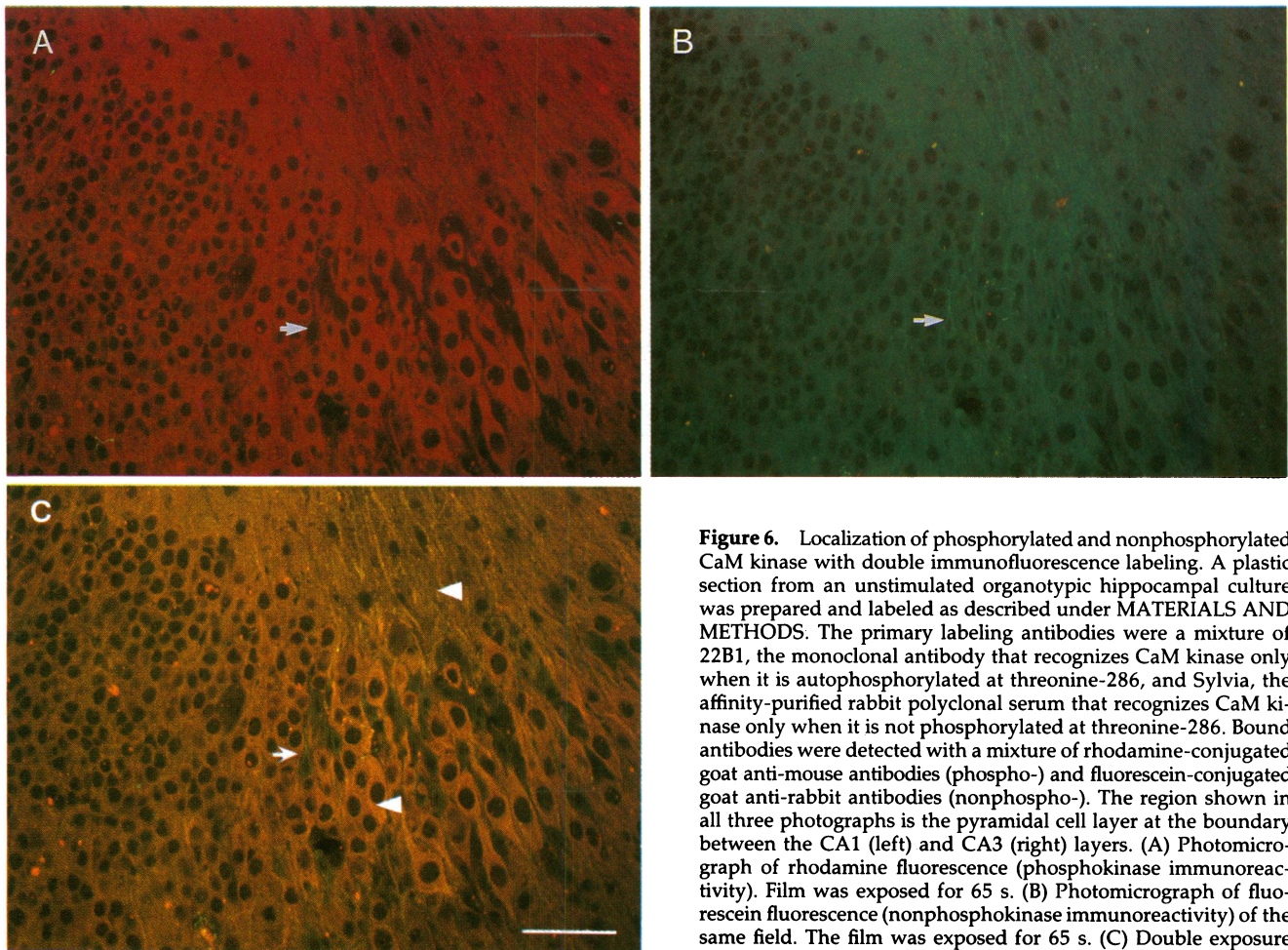


Figure 6. Localization of phosphorylated and nonphosphorylated CaM kinase with double immunofluorescence labeling. A plastic section from an unstimulated organotypic hippocampal culture was prepared and labeled as described under MATERIALS AND METHODS. The primary labeling antibodies were a mixture of 22B1, the monoclonal antibody that recognizes CaM kinase only when it is autophosphorylated at threonine-286, and Sylvia, the affinity-purified rabbit polyclonal serum that recognizes CaM kinase only when it is not phosphorylated at threonine-286. Bound antibodies were detected with a mixture of rhodamine-conjugated goat anti-mouse antibodies (phospho-) and fluorescein-conjugated goat anti-rabbit antibodies (nonphospho-). The region shown in all three photographs is the pyramidal cell layer at the boundary between the CA1 (left) and CA3 (right) layers. (A) Photomicrograph of rhodamine fluorescence (phosphokinase immunoreactivity). Film was exposed for 65 s. (B) Photomicrograph of fluorescein fluorescence (nonphosphokinase immunoreactivity) of the same field. The film was exposed for 65 s. (C) Double exposure (65 s each) of rhodamine and fluorescein fluorescence. Similar results were obtained in three experiments. Arrows, cells containing

no detectable phosphokinase immunoreactivity; upper arrowhead, neuropil region containing relatively less phosphokinase immunoreactivity; lower arrowhead, cell body layer containing relatively more phosphokinase immunoreactivity. Scale bar is 110 μ M.

DISCUSSION

In the study reported here, we examined the localization of autophosphorylated brain type II CaM kinase in organotypic hippocampal cultures. The kinase is activated in vitro by a rise in Ca^{2+} concentration; however, autophosphorylation of threonine-286 in the regulatory region causes it to remain active after the Ca^{2+} concentration has returned to basal levels (Miller and Kennedy, 1986; Miller *et al.*, 1988; Hanson *et al.*, 1989). Autophosphorylation of this same residue dramatically enhances the affinity of the kinase for calmodulin, perhaps influencing the distribution of the calmodulin pool (Meyer *et al.*, 1992). To better understand the role of the CaM kinase in regulating synaptic transmission, we are studying how its autophosphorylation is regulated in situ in hippocampal neurons.

We immunized mice with a 14-residue peptide with the sequence surrounding threonine-286 to generate

mouse monoclonal antibodies that bind to the CaM kinase only when it is phosphorylated at that threonine residue. Before immunization, the peptide was enzymatically thiophosphorylated, and the thiophosphopeptide was purified and conjugated to KLH (Patton *et al.*, 1991). This method yielded antibodies with high affinity and specificity for CaM kinase phosphorylated at threonine-286, although the yield of specific hybridomas was low (<2% of all hybridomas). We prefer to use phosphatase-resistant thiophosphorylated peptides, rather than phosphorylated peptides, as immunogens because they are resistant to dephosphorylation by the most common cellular phosphatases. Although phosphorylated peptides have been used successfully as immunogens by others, their efficiency as antigens was not reported. Lee *et al.* (1988) raised monoclonal antibodies against phosphorylated neurofilament proteins with either phosphorylated protein or phosphopeptides

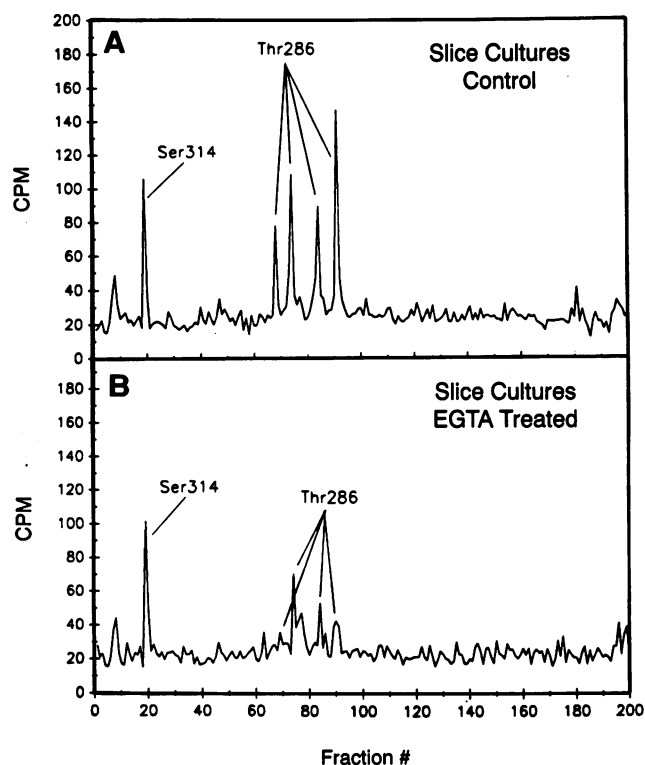


Figure 7. Removal of Ca^{2+} from the culture medium specifically reduces autophosphorylation of threonine-286. Two groups of five organotypic cultures were labeled with $^{32}\text{P}\text{-PO}_4$ for 8 h as described in Molloy and Kennedy (1991), except that 100 μM inorganic phosphate was included in the labeling Eagle's basal medium with Earle's Salts (BME/E) medium. After the labeling period, one group of cultures (Control) was incubated for 30 min in buffered saline (Molloy and Kennedy, 1991) containing 2 mM Ca^{2+} ; the other group (EGTA treated) was incubated in the same saline without Ca^{2+} and containing 200 μM EGTA. At the end of 30 min, the two groups of cultures were frozen and homogenized; anti-CaM kinase rabbit antiserum was added to the homogenates and the CaM kinase was precipitated with protein-A Sepharose CL-4B as described in Molloy and Kennedy (1991). Tryptic phosphopeptide maps were prepared as described in Patton *et al.* (1990). Tryptic phosphopeptides previously found to contain phosphorylated threonine-286 (Thr286) or serine-314 (Ser314) are indicated.

as antigen. Nishizawa *et al.* (1991) raised polyclonal antisera specific for phosphorylated intermediate filament protein by immunizing with synthetic peptides phosphorylated by the cyclic AMP-dependent protein kinase. It appears that thiophosphorylated and phosphorylated synthetic peptides will be generally useful for production of antibodies directed against identified functionally significant phosphorylation sites. A disadvantage of their use is that the synthetic peptide must be enzymatically phosphorylated by relatively large quantities of an identified protein kinase. However, newly emerging chemical methods for de novo synthesis of peptides containing phosphoamino acids should eliminate the need for large quantities of kinases (Perich

and Johns, 1988; Arendt *et al.*, 1989; Kitas *et al.*, 1990; Lacombe *et al.*, 1990).

Suzuki *et al.* (1992) used an immunization strategy similar to ours (Patton *et al.*, 1991) to produce a rabbit antiserum specific for CaM kinase phosphorylated at threonine-286, except that the peptide antigen was phosphorylated rather than thiophosphorylated. Staining of dissociated cultured hippocampal neurons with this antiserum was enhanced after treatment of the neurons with *N*-methyl-D-aspartate (NMDA). The biological significance of this result is not clear, however, because the neurons were treated with toxic doses of NMDA and fixed after 15 min when considerable excitotoxic damage would already have occurred. In addition, as noted in the INTRODUCTION and in Molloy and Kennedy (1991), autophosphorylation of CaM kinase II appears to be regulated differently in hippocampal neurons within organotypic cultures than in dissociated cultured hippocampal neurons.

To detect small differences in the proportion of autophosphorylation of CaM kinase II *in situ*, we raised a complementary rabbit polyclonal serum that binds to CaM kinase only when it is not phosphorylated at threonine-286 for use in double-label immunocytochemistry. To improve the specificity of the rabbit serum, antibodies that recognize nonphosphorylated CaM kinase were affinity-purified on a column substituted with the peptide antigen. The affinity purified antibodies bind weakly to a 65-kDa protein on immunoblots of brain homogenates as well as to nonphosphorylated kinase (Figure 3C). However, binding to the 65-kDa protein is not reduced by phosphorylation of the homogenate.

Our immunocytochemical results revealed that most neurons in the cultures contain both phosphorylated and nonphosphorylated kinase (Figures 5 and 6). Autophosphorylated kinase is not concentrated in a subpopulation of neurons as predicted by an early version of the "switch hypothesis" (Lisman, 1985). Our finding is consistent with earlier biochemical measurements that show that ~30% of the CaM kinase in such cultures is autophosphorylated at threonine-286/287 even though the basal Ca^{2+} concentration in the cultured neurons was only 15–45 nM (Molloy and Kennedy, 1991) (see also Figure 7). The nearly uniform distribution of autophosphorylated CaM kinase supports the hypothesis that the proportion of autophosphorylated CaM kinase at resting Ca^{2+} concentration is determined by a simple dynamic equilibrium between Ca^{2+} -stimulated autophosphorylation and dephosphorylation by protein phosphatases (Molloy and Kennedy, 1991). A subtle paradigm shift is occurring in our understanding of the regulatory roles of protein phosphorylation in general and of CaM kinase II in particular. It generally has been assumed that kinases work by being off most of the time and turned on for brief periods by physiological stimuli. However, taken together, the results in this ar-

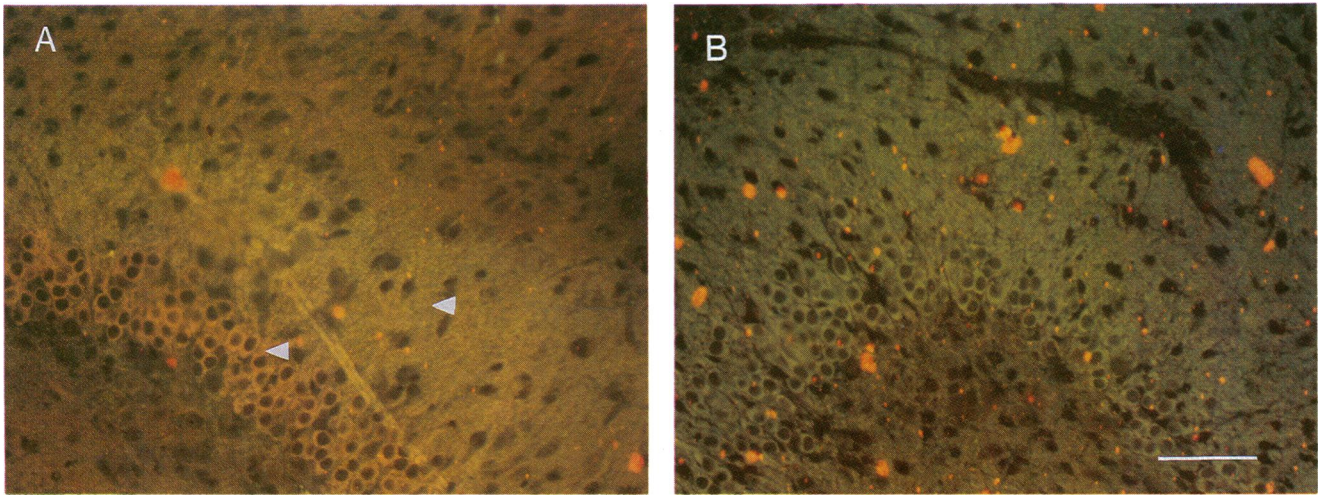


Figure 8. Removal of Ca^{2+} from the culture medium reduces phosphokinase immunoreactivity. Organotypic hippocampal cultures were placed in a buffered saline solution containing 200 μM EGTA for 30 min and then fixed and sectioned as described under MATERIALS AND METHODS. Sections were labeled with anti-phosphokinase and anti-nonphosphokinase as described in Figure 6. Control cultures not incubated in EGTA were sectioned and labeled in parallel. The areas pictured are from the dentate gyrus. (A) Double-exposed photomicrograph of a control culture showing overlapping distribution (orange to yellow hues) of nonphosphokinase and phosphokinase as in Figure 6. Upper arrowhead, neuropil region containing relatively less phosphokinase immunoreactivity; lower arrowhead, cell body layer containing relatively more phosphokinase immunoreactivity. (B) Double-exposed photomicrograph of an EGTA-treated culture taken with the same exposure times as in A. Although fluorescein fluorescence (nonphosphokinase immunoreactivity) is strong, rhodamine fluorescence (phosphokinase immunoreactivity) is dramatically reduced compared with the control. Orange particles are flakes of sealant present above the focal plane of the sections. Similar results were obtained in two experiments. Scale bar is 110 μM .

ticle and biochemical results reported elsewhere (i.e., Molloy and Kennedy, 1991; Ocorr and Schulman, 1991) indicate that CaM kinase II activity throughout most neurons in hippocampal organotypic cultures is maintained at an intermediate level by continuing autophosphorylation. Maintenance of this equilibrium is clearly expensive to the neuron and therefore must have produced a strong evolutionary advantage. We believe that the high basal activity reflects participation of the kinase in a network of interacting regulatory molecules that can respond to many stimuli. It will be important to establish how this high steady-state activity is distributed and controlled in neurons in different physiological situations.

Phosphorylated and nonphosphorylated CaM kinase molecules were colocalized throughout somas and dendrites in most neurons in the cultures that we examined. However, a small population of neurons (<2%), usually located at the CA1-CA3 border, appeared to contain only nonphosphorylated kinase (Figure 6, B and C, arrows). We do not yet know whether the absence of autophosphorylated kinase in these cells is biologically significant. In addition, neuronal somas appeared to contain a higher proportion of autophosphorylated kinase than did dendrites (compare upper and lower arrowheads in Figures 6C and 8A). Preliminary results reveal a similar distribution of phospho- and nonphosphokinase in sections of adult rat hippocampus (Hunt,

personal communication). The equilibrium steady state may be shifted slightly more toward autophosphorylated kinase in the soma than it is in dendrites, either by a higher basal Ca^{2+} concentration or by a lower concentration of active protein phosphatase.

Removal of Ca^{2+} from the culture medium causes dephosphorylation of the CaM kinase in situ; the overall proportion of autophosphorylated kinase is reduced from ~30% to 5–10% (Molloy and Kennedy, 1991). Similarly, staining with the phosphokinase-specific antibody 22B1 was dramatically reduced in cultures that had been incubated in EGTA. The results showed that the proportion of autophosphorylated kinase was reduced equally in somas and dendrites of all neurons (Figure 8B). This experiment also demonstrates unequivocally that autophosphorylation of the kinase enhances binding of 22B1 in tissue sections as well as on immunoblots. It reveals the potential sensitivity of the double-label fluorescence method; dephosphorylation of ~25% of the CaM kinase molecules was easily detected.

Biochemical studies of neurons have identified a large number of specialized neural proteins that are regulated by phosphorylation, and hypotheses about their physiological functions have been formulated (Nestler and Greengard, 1984; Kennedy, 1989; Skene, 1989; DeCamilli *et al.*, 1990). To test hypotheses about the functions of protein phosphorylation in the nervous system,

it would be useful to be able to measure the time course and extent of phosphorylation of identified neural phosphoproteins in intact tissue after physiological manipulations. However, such tests are usually technically difficult for several reasons. Because of the complex anatomy of the brain, it is often impossible to measure protein phosphorylation *in situ* within individual defined neuronal cell types or in specialized subcellular domains. Furthermore, protein phosphorylation in cells is inherently "noisy"; most proteins can be phosphorylated at relatively low rates at several sites. Such "non-specific" phosphorylation, which is usually functionally irrelevant, can overshadow changes in phosphorylation of functionally significant sites. Phosphorylation of individual sites can be detected after a protein phosphorylated in a living cell is fragmented into phosphopeptides. However, phosphopeptides can only be generated and measured easily when the protein is relatively abundant in a tissue homogenate, and such measurements usually provide at best only a semi-quantitative estimate of phosphorylation of a specific site. The sensitive immunocytochemical technique that we have used for visualizing autophosphorylation of the CaM kinase may be generally useful for visualization of individual sites on identified proteins in fixed tissue sections. We are now adapting the technique for use with confocal laser scanning microscopy so that we can obtain high resolution measurements of fluorescence emission without embedding the tissue in plastic. This adaptation will permit more quantitative analysis of the proportion of autophosphorylated kinase after physiological manipulations and eventually of other phosphorylated proteins in different parts of neurons.

ACKNOWLEDGMENTS

We thank Susan Ou and Julianna Horn-Krist of the Caltech Monoclonal Antibody Facility for their assistance and advice in producing monoclonal antibodies, and Leslie Schenker for expert technical assistance. Peptide synthesis was performed by Lisa L. Chang and Suzanna J. Horvath of the Caltech Biopolymer Synthesis Center. The pcDL-SR α 2 expression vector and mutant α subunit constructs were gifts from Robert Bulleit and Masamichi Ito. Financial support was provided by a grant from the Beckman Institute at Caltech, by NIH grant NS-17660 to M.B.K. and training grant NS07251, the Irvine Foundation, the McKnight Foundation, the Gustavus and Louis Pfeiffer Foundation, and the Joseph W. Drown Foundation. B.L.P. was supported in part by a Lawrence L. Ferguson Fellowship at Caltech.

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